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EXPERIMENTAL INDUCTION OF ODONTOBLAST DIFFERENTIATION AND STIMULATION DURING REPARATIVE PROCESSES

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Abstract

In vivo implantation experiments have shown that ethylenediaminetetraaceticacid(EDTA)-solublefractions of dentin stimulate reparative dentinogenesis. When isolated embryonic dental papillae were cultured in the presence of these dentin constituents, odontoblast cytological and functional differentiation could be initiated and maintained in the absence of an enamel organ. These effects were attributed to the presence of TGF-β-related molecules [TGF-β1 or bone morphogenetic protein-2a (BMP-2a)] which had to be used in combination with an EDTA-soluble fraction of dentin in order to specifically affect competent preodontoblasts. These EDTA-soluble constituents present in dentin could be replaced by heparin or fibronectin which both have been reported to interact with TGF-β. The association of such defined matrix components with a TGF-β-related molecule represents a biologically active complex triggering odontoblast functional differentiation.

In response to caries, odontoblasts modulate their secretory activity and are stimulated to elaborate reactionary dentin. This might be induced by active molecules such as IGF, TGF-6 or BMP which are liberated from dentin consecutively to the demineralization process.

Reparative dentinogenesis is distinct from reactionary dentinogenesis and more complex since it implicates the differentiation of precursor cells present in the dental papilla. The developmental history of these cells is different from that of the physiological predontoblasts in developing teeth. The nature of these "stem cells" and the mechanism of their induction still remain open questions.

Key Words: Odontoblast differentiation, polarization, dentin, transforming growth factor beta, fibronectin, extracellular matrix, cell-matrix interaction.

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Introduction

Tooth germs consist of two interacting tissues, the enamel organ and the dental papilla, with an interposed basement membrane. During tooth development, only the ecto-mesenchymal cells in contact with the basement membrane give rise to odontoblasts (Ruch *et al.*, 1982). However, all dental mesenchymal cells which derive from neural crest cells (Chibon, 1966, 1967; Lumsden, 1987; Smith and Hall, 1990) might be potential odontoblasts. This potentiality is probably expressed in reparative processes where dental mesenchymal cells can give rise to a second generation of odontoblasts (Schröder, 1985; Yamamura, 1985). The purpose of this review is to compare control mechanisms involved in the two processes of odontoblast differentiation and to see whether they may share some analogous steps.

Odontoblast Differentiation During Odontogenesis

Phenotypic aspects of odontoblasts differentiation

The combination of specific morphological, cytological and functional features determines the identity of odontoblasts (Ruch, 1985). Odontoblast terminal differentiation is characterized by a sequence of cytological and functional changes. First, preodontoblasts become postmitotic and during the last cell division, the mitotic spindle lies perpendicular to the basement membrane (Osman and Ruch, 1976; Ruch, unpublished observations). Only the daughter cells in contact with the basement membrane will become an odontoblast. These cells will then concomitantly elongate and polarize; these two events cannot be separated experimentally. It is the process of elongation and polarization that distinguishes a preodontoblast from an odontoblast. As such, these two characteristics provide us with the first markers of odontoblast differentiation and will be accompanied by the functional activities of the cell.

Polarization itself includes cytological and functional aspects. The cytological changes, which are realized in a few hours in the mouse embryo (Olive and Ruch, 1982b), have been described for many years and include the position of the nucleus at the basal pole of

the differentiated cell, the development of the ergastoplasmic cisternae which align parallel to the long axis of the odontoblast, changes in the distribution of the organelles, the development of junctional complexes, and the formation of a cell process (Garant and Cho, 1985; Takuma and Nagai, 1971). Cytoskeletal elements are involved in these changes; the use of either cytochalasin B or colchicin blocks the cytological and functional differentiation of odontoblasts (Ruch *et al.*, 1975). Microtubules (Nishikawa and Kitamura, 1987), intermediate filaments (Fausser *et al.*, 1990; Lesot *et al.*, 1982) and microfilaments (Kubler *et al.*, 1988; Lesot *et al.*, 1982; Nishikawa and Kitamura, 1986; Ruch *et al.*, 1987) are reorganized during odontoblast elongation and polarization. Odontoblast polarization has been suggested to result from the existence of a surface to which pulp cells might attach (Veis, 1985a). A polarity may already exist before the cell elongates. For example, the basement membrane induces a polarity in the preodontoblast which results in the specific orientation of the mitotic spindle during the last cell division (Osman and Ruch, 1976; Ruch, unpublished observations).

The functional aspects of odontoblast differentiation have also been extensively studied in several laboratories (Butler *et al.*, 1992; Linde, 1989; Veis, 1985b) and have demonstrated qualitative and quantitative metabolic changes leading to the polarized secretion of predentin-dentin which accumulates at the apical pole of odontoblasts, at the epithelio-mesenchymal junction. Functional odontoblasts synthesize collagens type I, type I trimer (Lesot, 1981; Lesot and Ruch, 1979; Munksgaard *et al.*, 1978; Wohllebe and Carmichael, 1978), type V and type VI (Becker *et al.*, 1986; Bronckers *et al.*, 1986). Functional odontoblasts also accumulate non-collagenous proteins such as osteocalcin (Bronckers *et al.*, 1987; Gorter de Vries *et al.*, 1988; Mark *et al.*, 1988), phosphoproteins (Butler, 1984; Butler *et al.*, 1983; Stetler-Stevenson and Veis, 1983; Takagi and Sasaki, 1986), acidic glycoproteins (Butler *et al.*, 1981; Fisher *et al.*, 1983) and proteoglycans (Goldberg and Escaig, 1985; Hjerpe *et al.*, 1983; Takagi *et al.*, 1990). It is now apparent that dentin also contains several growth factor-like molecules. Although their secretion by odontoblasts remains to be demonstrated, it is probable that these molecules originate, at least in part, from the odontoblasts.

Control of odontoblast differentiation

Heterochronal recombinations of dental tissues and tritiated thymidine incorporation (Ruch *et al.*, 1976), as well as the comparison of cell proliferation kinetics during odontogenesis *in vivo* and *in vitro* (Ahmad and Ruch, 1987), have provided phenomenological support to the hypothesis suggesting that terminal differentiation of odontoblasts (and ameloblasts) could be triggered by specific epigenetic signals only after a minimal number of cell cycles. These signals are provided by epithelio-mesenchymal, matrix-mediated interactions (Koch, 1967; Kollar, 1983; Ruch, 1985, 1987; Ruch *et al.*, 1982; Slavkin, 1978; Thesleff and Hurmerinta, 1981; Thesleff *et al.*, 1989). The terminal differentiation of odontoblasts requires the presence of a stage specific basement membrane (Kollar, 1983; Lumsden, 1987; Meyer *et al.*, 1977; Osman and Ruch, 1981; Ruch *et al.*, 1982, 1983; Slavkin, 1990; Slavkin *et al.*, 1988). This basement membrane might act either as a specific substrate (Lesot *et al.*, 1981, 1985b, 1992; Mark *et al.*, 1990) or as a reservoir of paracrine and autocrine factors (Cam *et al.*, 1992; Ruoslahti and Yamaguchi, 1991; Schubert, 1992). The dental basement membrane might thus participate in the control of both cell proliferation kinetics (Olive and Ruch, 1982a) and cytodifferentiation.

Among the constituents of the basement membrane, fibronectin was shown to be redistributed during odontoblast polarization (Lesot *et al.*, 1981; Thesleff and Hurmerinta, 1981) and thus was suspected to play a role in the control of odontoblast differentiation. Several reports have shown that this molecule could interact with cell surfaces for example by means of integrins (Hynes, 1992) and that the β1 subunit of integrins could interact with the microfilament system by means of either talin (Horwitz *et al.*, 1986) or, more probably, α-actinin (Otey *et al.*, 1990). Fibronectin was found to interact with dental mesenchymal cell surfaces by means of three high molecular weight membrane proteins (Lesot *et al.*, 1985a). At least one of these proteins, with a molecular weight of 165 kDa, is expressed by odontoblasts and was found to play a role in the reorganization of microfilaments during odontoblast polarization; a monoclonal antibody directed against an extracellular epitope of this protein inhibited odontoblast differentiation *in vitro* (Lesot *et al.*, 1988). This 165 kDa protein has a transitory expression localized at the apical pole of newly differentiated odontoblasts; the 165 kDa protein rapidly disappeared at a stage where fibronectin also could no longer be detected in this region (Lesot *et al.*, 1990). At this stage, the formation of junctional complexes including tight junctions, zonulae adherens, and gap-junctions increases (Bishop, 1985; Callé, 1985; Iguchi *et al.*, 1984). Gap-junctions might be involved in the control of the functional state of polarized odontoblasts.

Several complementary data have indicated that the 165 kDa protein is not related to integrins. After staining of tooth germs with antibodies directed against the integrin β1 chain, odontoblasts remained negative although the 165 kDa protein could be detected (Lesot *et al.*, 1992). Staining of cultured dental mesenchymal cells for the two antigens demonstrated different localizations (Lesot *et al.*, 1992). Among eight integrins which interact with fibronectin, six recognize a specific site on the molecule which involves the GRGDS sequence (Hynes, 1992). GRGDS(P) synthetic peptides did not inhibit odontoblast differentiation (Lesot *et al.*, 1992) although the use of a monoclonal antibody directed against the 165 kDa protein completely inhibited the process (Lesot *et al.*, 1988). Finally, the site mediating

interaction with the 165 kDa protein has been localized close to the collagen binding site of fibronectin and implicated the III-1 repeat unit of the molecule (Lesot *et al.*, 1992). No integrin interacts with this region of fibronectin.

These biochemical results were confirmed by culturing first lower molars, obtained from day-16 mouse embryos, for four days in the presence of synthetic GRGDS peptides (up to 800 μg/ml). Odontoblasts normally differentiated (Fig. 1c-d) as in control cultured tooth germs (Fig. 1a-b). These observations confirmed the fact that odontoblast polarization was found to be a RGD-independent process (Lesot *et al.*, 1992). GRGDS peptides only affected the interaction of the inner dental epithelium (preameloblasts) with the basement membrane (Fig. 1e) and the processing of this basement membrane which appeared duplicated (Fig. 1e). Control experiments performed with GRGES synthetic peptides demonstrated a normal aspect of the basement membrane which was never duplicated (not shown). Immunofluorescent staining of mouse tooth germ frozen sections with anti-integrin antibodies revealed positive staining of the inner dental epithelium with anti-β4 (Fig. 2a) or anti-α6 (Fig. 2c), whilst no reaction was observed when using anti-β1 antibodies (Fig. 2e). Although the specificity of $\alpha 6\beta 4$ integrin is controversial (Lotz *et al.*, 1990; Sonnenberg *et al.*, 1991), it has been reported to mediate interaction with laminin (Lee *et al.*, 1992). Again, this interaction appears to be RGD-independent (Hynes, 1992), suggesting the existence of other integrins mediating interactions in between the inner dental epithelium and basement membrane constituents.

Effects of matrix molecules

The presence of extracellular matrix molecules and/or paracrine/autocrine factors in the basement membrane may provide the necessary inductive signal for odontoblast differentiation. Although the identity of the molecule(s) responsible for this inductive signal are still under investigation, it is possible that the requirement for a specific basement membrane might be experimentally replaced by substitution of immobilized matrix molecules.

First molars were obtained from mouse embryos at day 18; at this stage the first odontoblasts at the tip of the main cusps are post-mitotic and engaged in their terminal differentiation. These tooth germs have been enzymatically dissociated and isolated dental papillae were cultured for three days on Millipore filters coated with either fibronectin, collagen type I, or fibronectin and collagen type I (Lesot *et al.*, 1985b). Despite possible interactions of collagen type I and fibronectin with the surfaces of preodontoblasts-odontoblasts (Lesot *et al.*, 1985a), none of these substrates were found to promote odontoblast elongation and polarization (Lesot *et al.*, 1985b). The maintenance of odontoblast polarization could be observed when dental papillae were cultured either on Millipore filters which had been coated with a biomatrix extracted from dental papillae (Cam *et al.*,

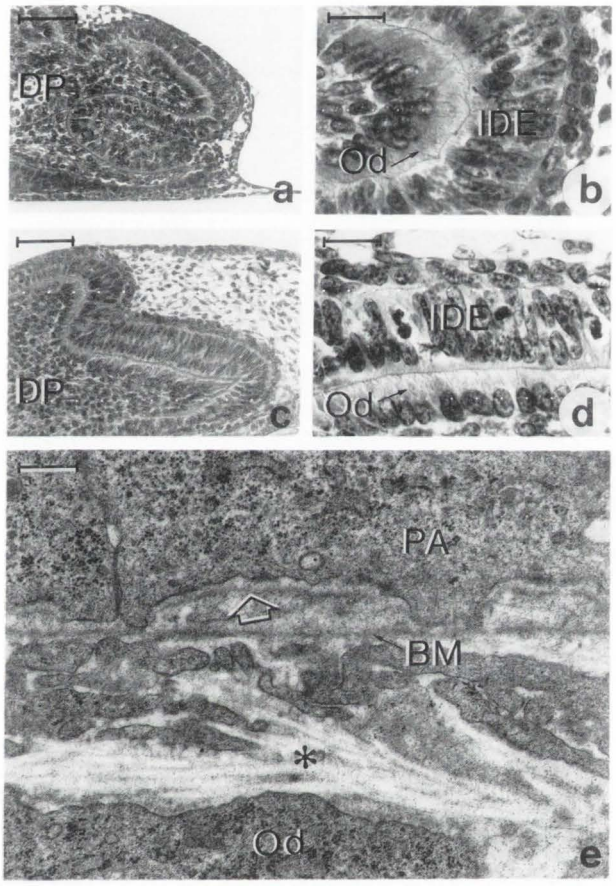


Figure 1. **(a-d)** Histological sections of day-16 mouse tooth germs cultured for 4 days in control medium (a,b) or in the presence of 800 μg/ml GRGDS peptide (c,d). In both cases, polarized odontoblasts (Od) were facing preameloblasts (PA) in the inner dental epithelium (IDE). Bar = 15 μm (a, c), 70 μm (b, d). **(e)** Transmission electron microscopy demonstrated that the GRGDS peptides (e) interfered with the interaction of the inner dental epithelium with the basement membrane (BM). The basement membrane appeared duplicated (open arrow). Odontoblasts (Od) were functional and accumulated collagen (*) at the epithelio-mesenchymal junction. Bar = 0.5 μm.

Tooth germs were cultured on Millipore filters in RPMI-1640 medium supplemented with 15% fetal calf serum (FCS). The culture medium was changed every two days. Cultures were incubated at 37°C in humidified 5% CO_2 in air. Cultured tooth germs were either fixed in Bouin's fixative, processed for histology and stained with haemalun eosin, or fixed in a 2% glutaraldehyde solution buffered with 0.1 M cacodylate, pH 7.3, post-fixed in 1% osmium tetroxide in the same buffer and processed for electron microscopy (Meyer *et al.*, 1977).

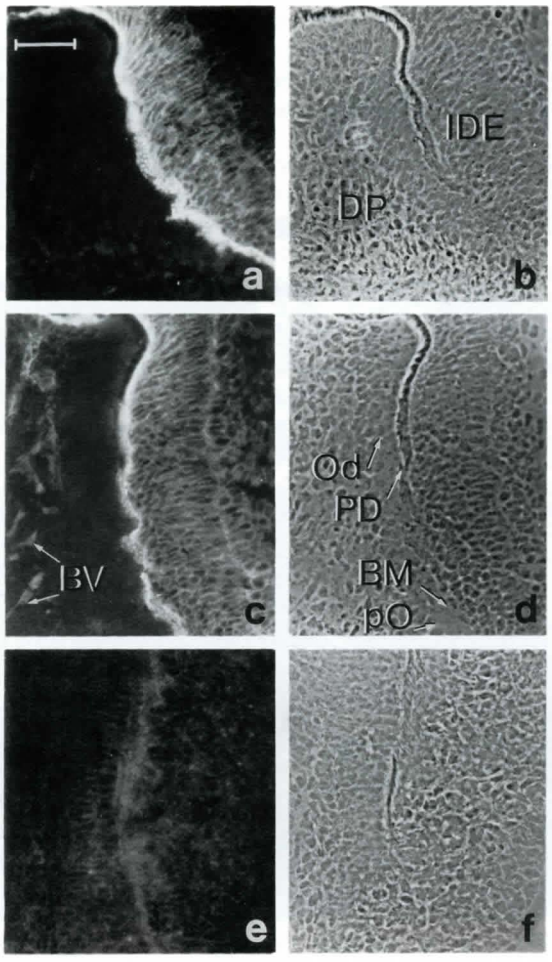


Figure 2. Indirect immunofluorescence (**a, c, e**) and phase contrast microscopy (**b, d, f**) of day-20 mouse tooth germs frozen sections. Sections were fixed with 3% formaldehyde and stained with antibodies directed against integrin subunit $\beta 4$ (Kennel *et al.*, 1989), $\alpha 6$ (Kennel *et al.*, 1989), and $\beta 1$ using the conditions described by Hertle *et al.* (1991). Preodontoblasts-odontoblasts remained unstained although the $\beta 4$ (a) and the $\alpha 6$ (c) subunits were localized in the inner dental epithelium. Antibodies directed against the $\beta 1$ chain did not stain dental tissues (e). Bar = 40 μm.

1986) or on uncoated Millipore filters when hyaluronic acid or chondroitin sulfate was added to the culture medium (Tziafas *et al.*, 1988).

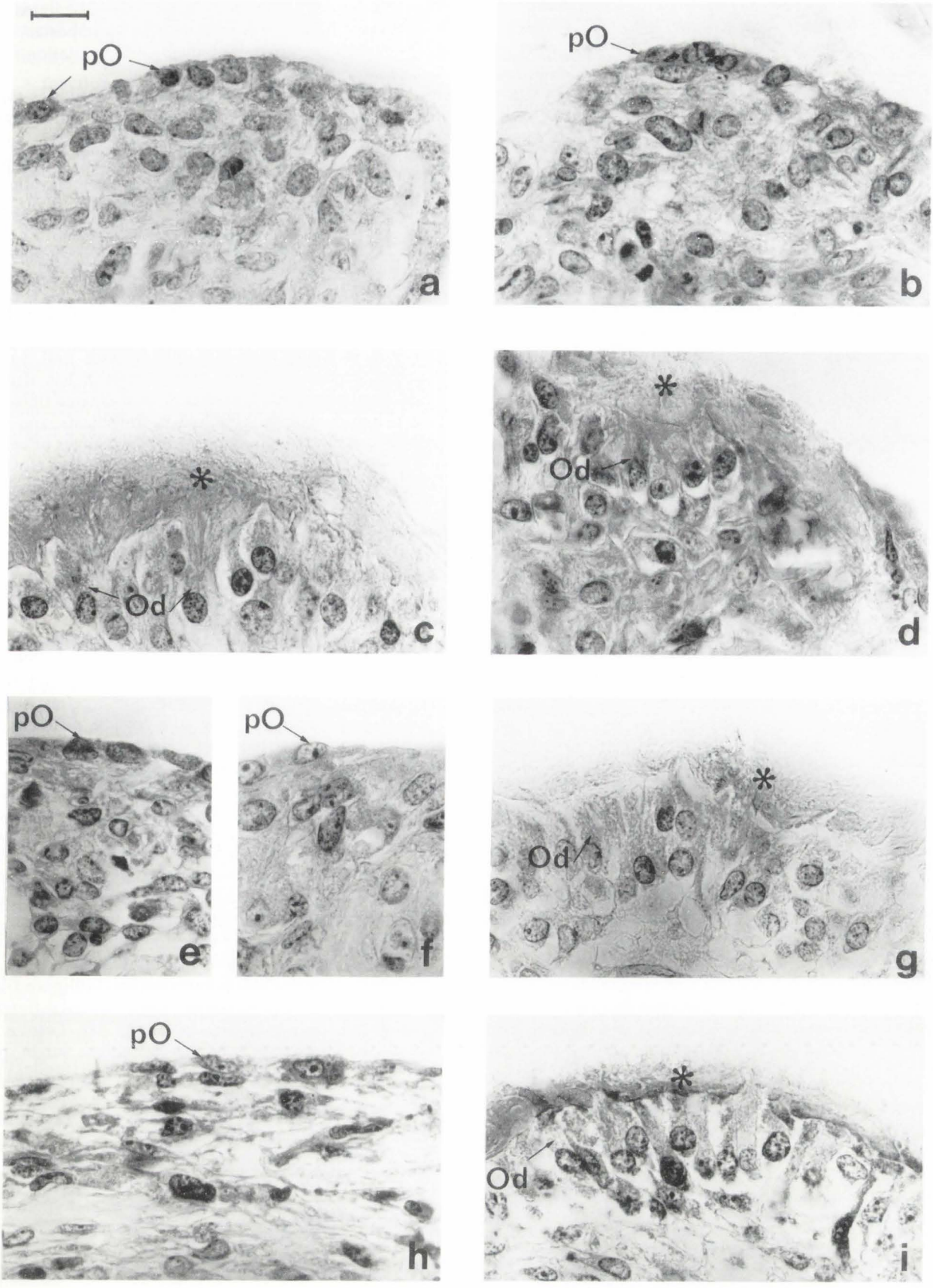
Odontoblasts differentiation can also occur in the absence of a specific basement membrane, for example, during reparative processes. In such situations, contact with dentin matrix appears important for odontoblast differentiation and it is possible that molecules present in dentin provide the inductive signal in much the same manner as the basement membrane during odontogenesis. Thus, experimentally replacing the dental basement membrane by the molecules normally found therein or within the dentin matrix allowed complementary investigations to study the control mechanisms involved in odontoblast differentiation in cultures of isolated embryonic dental papillae.

Figure 3 (on the facing page). Trypsin isolated day-17 dental papillae were embedded in 12 μl of a semi-solid medium containing 0.5% agar and cultured for 6 days on Millipore filters as described in the legend of Fig. 1 (see also Bègue-Kirn *et al*, 1992). To test their biological effects, different substrates were included in the agar (**b-g**) and compared to control conditions with agar only (**a**). The different substrates were 3.6 μg of the total EDTA-soluble fraction of dentin (**b**), 3.6 μg of EDTA-soluble constituents of dentin retained on DEAE-Cellulose (**c**), 50 ng TGF-$\beta 1$ in the presence of 3.6 μg of the total EDTA soluble fraction of dentin (**d**), 1.2 μg of heparin (**e**), 50 ng TGF-$\beta 1$ alone (**f**), 50 ng TGF-$\beta 1$ in the presence of 1.2 μg of heparin (**g**), 0.5 μg of fibronectin (**h**), and 50 ng TGF-β in the presence of 0.5 μg of fibronectin (**i**).

Cells at the periphery of the explant (preodontoblasts) polarized and gave rise to functional odontoblast-like cells when grown in agar containing the EDTA soluble fraction of dentin retained on DEAE-Cellulose (c) but never differentiated in control medium (a), in the presence of heparin (e), in the presence of fibronectin (h) nor in the presence of the total EDTA-soluble fraction of dentin (b). Whilst TGF-$\beta 1$ alone stimulated the secretion of extracellular matrix (*) throughout the dental papilla, cells at the periphery did not polarize (f). TGF-$\beta 1$ in combination with either the inactive fraction of dentin (d), heparin (g) or fibronectin (i) promoted the elongation and polarized secretion of extracellular matrix by odontoblast-like cells (Od) at the periphery of the explants. pO: preodontoblasts. Bar = 15 μm.

Dentin matrix components were isolated from rabbit dentin after exhaustive extraction with ethylenediaminetetraacetic acid (EDTA) in the presence of protease inhibitors followed by digestion of the insoluble matrix with collagenase (Smith and Leaver, 1979, 1981; Smith *et al.*, 1980). Dental papillae from day-18 tooth germs have thus been cultured on Millipore filters coated with various isolated dentin matrix fractions (Lesot *et al.*, 1986). These experiments demonstrated that, despite the absence of enamel organ and basement membrane, the odontoblasts, which were polarized at the onset of the culture, could maintain their elongated shape when grown on two specific fractions. One active fraction was EDTA-soluble and the other was only released after collagenase treatment.

However, the initiation of odontoblasts polarization was never observed in these conditions (Lesot *et al.*, 1986). This may have been in part due to technical difficulties in providing optimal conditions for interactions between the dental papillae cells and the immobilized matrix components. More recently, trypsin-isolated day-17 mouse molar dental papillae (containing


pO
a
pO
b
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Od
c
*
Od
d
pO
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f
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only preodontoblasts) were cultured for six days in semi-solid agar medium containing the same EDTA-soluble dentin matrix fractions separated by ion-exchange chromatography as described above. The total unpurified EDTA-soluble fraction neither promoted nor maintained odontoblast differentiation (Fig. 3b) and only undifferentiated cells were observed at the periphery of the explant. The appearance of these cells was similar to those of control cultures (Fig. 3a) performed in the absence of dentin fractions. However, dentin matrix fractions retained on DEAE (diethylaminoethyl)-Cellulose (Bègue-Kirn *et al.*, 1992) were observed to initiate the differentiation of odontoblast-like cells at the periphery of the explants (Fig. 3c). In these conditions, the normal pattern of a gradient of odontoblast differentiation could be seen in the dental papillae with initiation at the tips of the main cusps and a progression in an apical direction (as in cultured intact day-17 molars). Collagen type I and fibronectin were found to be constituents of the extracellular matrix which accumulated at the secretory pole of the elongated cells.

Effects of matrix-associated molecules

The active components present in the EDTA-soluble fraction of dentin could be retained on heparin-Agarose columns. This affinity for heparin-Agarose is shared by several growth factors, including TGF-β. In view of the possible role for TGF-β in tooth development, investigations were directed towards possible relationships between isolated dentin matrix fractions and molecules of the TGF-β family.

Addition of a blocking TGF-β antibody (Dasch *et al.*, 1989) to the culture medium abolished the biological effects of the active dentin matrix fraction in odontoblast differentiation (Bègue-Kirn *et al.*, 1992). This result led to investigation as to whether molecules of the TGF-β family, either alone or in combination with the inactive unpurified total EDTA-soluble dentin matrix fraction, could initiate odontoblast differentiation in day-17 isolated mouse dental papillae. When either TGF-β1 (Gentry *et al.*, 1987) or bone morphogenetic protein-2 (BMP-2) (Wozney *et al.*, 1988) was added to the inactive total EDTA-soluble fraction of dentin constituents, the initiation of odontoblasts differentiation could also be induced (Fig. 3d) whilst TGF-β1 alone (Fig. 3f), or BMP-2 alone only stimulated a general production of extracellular matrix throughout the whole dental papilla cells; odontoblast-like cells were never observed in these conditions (Fig. 3f) (Bègue-Kirn *et al.*, 1992).

Inactive fractions (inactivation resulted from long term storage at 4°C instead of -20°C) of EDTA-soluble proteins could be replaced by heparin which is known to bind TGF-β (McCaffrey *et al.*, 1992; Rappolee *et al.*, 1988) and BMP-2 (Wang *et al.*, 1988). Although heparin alone had no effect on cultures of isolated dental papillae (Fig. 3e), the combination of heparin with TGF-β1 also induced the differentiation of odontoblast-like cells (Fig. 3g) at the periphery of the explants (Bègue-Kirn *et al.*, 1992).

Two types of molecules, TGF-β1-related molecules and fibronectin, seem to play an important role in the initiation of odontoblast differentiation during dentinogenesis.

TGF-βs influence several cell activities through complex interactions: TGF-βs affect cell behaviour through changes in the extracellular matrix, and the activity of TGF-βs can also be modulated by the composition of the matrix. TGF-β modulates the synthesis of collagens (Ignotz and Massagué, 1986; Yu *et al.*, 1991) and that of proteoglycans (Rapraeger, 1989; Yu *et al.*, 1991), two types of molecules which interact with fibronectin. Concerning fibronectin itself, TGF-β1 has been shown to interfere with its synthesis (Ignotz and Massagué, 1986; Williams and Allen-Hoffmann, 1990), alternative splicing (Magnuson *et al.*, 1991; Wang *et al.*, 1991), assembly and interaction with cell surfaces (Allen-Hoffmann *et al.*, 1988). TGF-β has also been shown to enhance the interaction of iodinated fibronectin to the surface of cultured human fibroblasts, and such a mechanism might be involved in the changes of cell-matrix interaction during morphogenesis (Allen-Hoffmann *et al.*, 1988). TGF-β can modulate the expression of integrins subunits (Sheppard *et al.*, 1992). On the other hand, TGF-β was reported to inhibit the degradation of matrix constituents (Edwards *et al.*, 1987; Laiho *et al.*, 1986). However, depending on its concentration, TGF-β can stimulate matrix synthesis or resorption (Hock *et al.*, 1990; Tashjian *et al.*, 1985).

Several transcripts coding for members of the TGF-βs superfamily (Burt, 1992; Massagué 1990) such as TGF-β1, TGF-β2, TGF-β3 or Vgr-1, and BMP-2 have been localized in developing tooth germs, suggesting a possible coordinated role for the different transcription products during odontogenesis (Hall and Ekanayake, 1991; Lehnert and Akhurst, 1988; Lyons *et al.*, 1990; Pelton *et al.*, 1989, 1990). At a stage which precedes odontoblast differentiation, cells from the inner dental epithelium (preameloblasts) might synthesize TGF-β1 which could then affect fibronectin synthesis by preodontoblasts-odontoblasts. This last step, implying interactions of fibronectin with the 165 kDa protein (Lesot *et al.*, 1988), would represent an autocrine control of the organization and/or function of the microfilament system in differentiating odontoblasts.

Fibronectin, when used to coat Millipore filters, neither promoted nor maintained odontoblast differentiation in cultures of isolated dental papillae (Lesot *et al.*, 1985b). However, technical limitations might result from the culture system since Tziafas *et al.* (1992a) have now demonstrated by *in vivo* experiments that fibronectin coated to Millipore filters could induce odontoblast differentiation when implanted in the dental papilla of dogs. More recently, Day-17 isolated dental papillae were cultured in agar containing fibronectin (Fig. 3h) or fibronectin and TGF-β1 (Fig. 3g); although again fibronectin alone had no effect (Fig. 3h), the differentiation of odontoblasts could be initiated in the presence of fibronectin and TGF-β1 (Fig. 3i). Again, these effects

could result from the ability of fibronectin to bind TGF-β (Fava and McLure, 1987; Mooradian *et al.*, 1989).

Working hypothesis

A functional network consisting of matrix molecules (including fibronectin) and growth factors (including TGF-β1) control odontoblast differentiation. TGF-βs or related molecules play a key role as shown by using blocking antibodies. Whilst TGF-β alone stimulated a general synthesis of extracellular matrix among dental papillae cells, the simultaneous presence of either heparin, or fibronectin in agar further allowed the elongation and cytological polarization of cells at the periphery of the explant and also an apical accumulation of matrix. The geometry of epithelially derived TGF-βs distribution, resulting from the presence of the physiological matrix (basement membrane) in developing tooth germs or imposed by heparin (or fibronectin) in cultured dental papillae, could lead to a polarized interaction of TGF-β with competent preodontoblasts. This interaction could then trigger a cascade of metabolic events, a reorganization of the cytoskeleton, and lead to an apical secretion of matrix (fibronectin) which would amplify and stabilize the polarization. We intend to test this hypothesis by checking whether or not TGF-β related molecules are physiologically involved in the regulation of odontoblast differentiation during odontogenesis and also by investigating the role of early responses to TGF-βs in cultured dental papillae.

Odontoblast Stimulation During Reparative Processes

In response to the demineralization process which accompanies caries, odontoblasts are stimulated to elaborate reactionary dentin. After an initial step of dentinal sclerosis, the metabolic activity (synthesis of collagen, alkaline phosphatase activity) of odontoblasts is enhanced. An increased secretion of collagen type III and fibronectin has been reported (Magloire *et al.*, 1988). Studying the local regulation of odontoblasts in this context, Magloire *et al.* (1992) have shown that the cells of the affected zone can be stained with anti-fibronectin antibodies, whilst in normal conditions odontoblasts would be negative. Furthermore, in response to a carious lesion, odontoblasts also re-express the 165 kDa membrane protein (Magloire *et al.*, 1992). Odontoblasts can modulate their secretory activity in response to the intensity of cariogenic stimulus. Variations in the regularity of dentinal tubule structure are well recognized and brief periods of intense matrix secretion can be observed histochemically (Perry and Smith, 1992).

Dentin has been shown to possess mitogenic activity (Finkelman *et al.*, 1990) and also to stimulate chondrogenesis and osteogenesis (Harada *et al.*, 1990; Rabinowitz *et al.*, 1990). The presence of bone morphogenetic activity in dentin has been reported (Bang and Urist, 1967; Butler *et al.*, 1977; Conover and Urist, 1979) and more recently, dentin constituents related to the bone morphogenetic proteins have been isolated (Bessho *et al.*, 1991; Kawai and Urist, 1989). Finkelman, *et al.* (1990) have shown that dentin was, like bone matrix, a potential source of several growth factors including IGF-I, SGF/IGF-II as well as TGF-β. These authors suggested that in pathological situations such as caries, growth factors could be liberated from the dentin during the demineralization process and thus stimulate adjacent odontoblasts to secrete reactionary dentin (Finkelman *et al.*, 1990). An EDTA-soluble fraction of dentin demonstrated a stimulatory effect on odontoblast activity when implanted in cavities without direct pulp exposure [Smith *et al.* (Smith AJ, Tobias RS, Cassidy, Plant CG, Browne RM, Bègue-Kirn C, Ruch JV, Lesot H. Odontoblast stimulation by dentine matrix components. Submitted to Archs. oral Biol.)]. Under such conditions, diffusion of the active fractions down the dentinal tubules could directly stimulate existing odontoblasts to secrete reactionary dentin (Smith *et al.*, submitted). These active fractions of dentin were the same as those found to allow the initiation and maintenance of odontoblast polarization *in vitro* (Bègue-Kirn *et al.*, 1992; Lesot *et al.*, 1986).

In such experiments, it is important to control the possible trauma to odontoblasts during cavity preparation, which itself could invoke a response. Use of control cavities in the ferrets without protein implantation, in our experiments (Smith *et al.*, 1990; submitted), showed little histological change in the pulp and no evidence for stimulation of odontoblast activity, thus confirming that trauma to the odontoblasts had been minimized. Despite probable damage to the odontoblast process during careful cavity preparation, when odontoblast traumatization is minimized, the cells were still able to function and secrete an extracellular matrix (Smith *et al.*, submitted).

Primary and reactionary dentin show structural and compositional differences (Magloire *et al.*, 1988; Perry and Smith, 1992; Schröder and Sundström, 1974). For example, fibronectin and collagen type III are constituents of reactionary dentin although they are absent from primary dentin (Magloire *et al.*, 1988), whilst phosphophoryn, a constituent of primary dentin, is largely absent from reactionary dentin (Takagi and Sasaki, 1986). Such observations demonstrate how odontoblasts can modulate their secretory activity in response to different environmental stimuli.

Reparative Dentinogenesis

Progressively during odontogenesis and at older stages, the neural crest-derived dental papilla cells (probably potential odontoblasts) express fibroblast-like phenotypes and non-dental cells (fibroblasts, vascular cells, histiocytes, macrophages, etc.) invade the dental papilla which constitutes a heterogeneous cell population; the odontoblast-like cells which are involved in reparative processes probably derive from the initial ecto-mesenchymal dental papilla cells. However, this is still controversial (Seltzer and Bender, 1984; Yamamura, 1985).

The evolution of reparative processes in the pulp tissues observed after destruction of primary odontoblasts has been extensively reviewed by Baume (1980). After operative procedures in clinical dentistry, the pulp response to damage in the dentin can result in destruction of odontoblasts. These are then replaced by odontoblast-like cells which could differentiate from pulpal cells (Sveen and Hawes, 1968; Fitzgerald *et al.*, 1990) and deposit reparative dentin (Bergenholtz, 1981; Trowbridge, 1981).

When calcium hydroxide is applied to an exposed pulp, a superficial necrosis develops, and it has been hypothesized that this causes slight irritation and thus stimulates defense and repair by the pulp (Schröder, 1985). Although the effects of calcium hydroxide are well documented (Baume, 1980; Cox *et al.*, 1982, 1985; Cvek *et al.*, 1987; Holland *et al.*, 1982; Seux *et al.*, 1991; Yamamura, 1985), the mechanism of action (chemical injury) is still unclear. A number of processes are taking place after application of calcium hydroxide and it is difficult to separate those which are beneficial from those which are harmful. After application of calcium hydroxide to exposed pulps, Schröder (1985) examined the sequence of events for up to six months in the human dental pulp. The reparative processes involved: (1) a proliferation and migration of the dental papillae cells, (2) the elaboration of a new collagenous matrix, (3) a dystrophic calcification of the area of necrosis, and (4) mineralization of the newly deposited collagen, which led to the formation of osteodentin or fibrodentin. Then a new generation of odontoblast-like cells differentiated and secreted dentin. It is interesting to speculate as to whether the direct effects of calcium hydroxide on the pulp represent induction of necrosis and are without benefit, whilst indirect effects of its pH might result in dissolution of growth factors from dentin in an analogous manner to that suggested by Finkelman *et al.* (1990) in caries leading to odontoblast differentiation.

Because of a potential clinical interest, most approaches are now directed towards the molecular mechanisms which initiate odontoblast differentiation in reparative processes. Several groups are investigating the biological properties of active molecules present in dentin.

Attempts have been made to see whether dentin matrix could stimulate or induce odontoblast differentiation by implanting either demineralized dentin in the dental papillae (Tziafas and Kolokuris, 1990) or non-collagenous fractions of dentin into the base of exposed and non-exposed cavities (Smith *et al.*, 1990; Smith *et al.*, submitted).

Demineralized bone or dentin, when implanted in dental papillae, can induce secondary dentinogenesis with deposition of osteodentin followed by tubular predentin formation (Tziafas and Kolokuris, 1990). Similar observations have been made when dentin chips were in contact with dental papillae cells (Selzer and Bender, 1984). All these results were obtained after rather long periods of implantation (two to four weeks). More recently, Tziafas *et al.* (1992b) analyzed the effects of dentin implantation in dental pulp after only three to ten days. The results led to the conclusion that differentiation of odontoblast-like cells induced by dentin resulted from a two-step mechanism: first dentin stimulates the synthesis of new matrix which then controls cell polarization (Tziafas *et al.*, 1992b).

The implantation of non-collagenous fractions of dentin into the base of exposed cavities in the ferret enhanced reparative dentinogenesis as compared to control cavities. Polarized, columnar odontoblast-like cells and tubular dentin formation were observed (Smith *et al.*, 1990). This could be observed both at the pulp-predentin interface and also, along the exposure walls perpendicular to the dentin matrix of the cavity base. Active fractions were obtained from dentin both after EDTA-extraction, and collagenase treatment of the insoluble residue (Smith *et al.*, 1990).

Millipore filters coated with fibronectin have also been shown to induce elongation and polarization of ecto-mesenchymal cells when implanted for one week in dog dental papillae (Tziafas *et al.*, 1992a). After four weeks, these odontoblast-like cells deposited calcified atubular and tubular dentinal matrix in contact with the filter (Tziafas *et al.*, 1992a). Although fibronectin can be involved in the process of odontoblast elongation and polarization (Lesot *et al.*, 1988), it is not clear yet how this cytological reorganization can be related to the functional differentiation of odontoblast (i.e., synthesis and secretion of dentin constituents). Since fibronectin from many sources has been shown to contain TGF-β activity (Fava and McClure, 1987) or can bind endogenous TGF-β, it would be of interest to determine, in these experimental conditions, if a synergy between the two types of molecules is necessary.

Active fractions of dentin proteins were microinjected in the dental papillae of day-18 tooth germs from mouse embryos in order to study the response of dental mesenchymal cells. Microinjected tooth germs were then cultured for 6 or 15 days. Tooth germs microinjected with active fractions of dentin proteins demonstrated an accumulation of extracellular matrix with included cells (Fig. 4a-c); however odontoblasts-like cells never differentiated, even after 15 days. This has demonstrated the ability of such dentin fractions to stimulate extracellular matrix synthesis by dental papillae cells and suggests that cells distant from the normal site of odontoblasts cannot differentiate because they are not competent to respond to the necessary signal. This may be due to their having not undergone the requisite number of cell divisions or for some other reason. When the microinjection was performed in close vicinity to the subodontoblastic layer, these cells never polarized (Fig. 4c). Technical limitations of immobilizing the dentin proteins may arise after microinjection, which could explain the above observation or else, the presence of surrounding cells could be exerting a negative type of control over the differentiation process.

TGF-β1 was also microinjected and the injection

site was visualized with Superose beads; important deposition of extracellular matrix was observed in association with the beads (Fig. 4d) but again polarized odontoblast-like cells were never observed. Thus, stimulation of extracellular matrix production by dental papillae cells was achieved, but differentiation could not occur.

Conclusions

During odontogenesis, the differentiation of odontoblasts, which never occurs spontaneously, is regulated in time and space by the inner dental epithelium through matrix-mediated interactions (Ruch and Karcher-Djuricic, 1971; Ruch *et al.*, 1976, 1978, 1982; Thesleff and Hurmerinta, 1981). In the absence of the inner dental epithelium, a specific basement membrane can initiate the polarization and functional differentiation of postmitotic preodontoblasts (Osman and Ruch, 1981).

The data summarized in this review have shown that the same fraction of EDTA-soluble constituents of dentin **1)** mimicked the effect of the inner dental epithelium on odontoblast terminal differentiation in cultures of isolated embryonic mouse dental papillae, and **2)** stimulated odontoblasts from adult ferrets to secrete reactionary dentin. The first observation refers to a complex mechanism leading from preodontoblasts to postmitotic and functional odontoblasts; the second point might be considered as a modulation of the functional state of odontoblasts.

The effects of active fractions of dentin on cultured embryonic dental papillae could be attributed to TGF-β1 as shown when using blocking antibodies directed against this molecule. Furthermore, TGF-β1 or BMP-2, when combined with either an inactive fraction of dentin or with heparin, could be used instead of active fractions of dentin since it also induced odontoblast differentiation.

It has been shown by *in situ* hybridization that TGF-β1 mRNA is expressed by the inner dental epithelium at a stage preceding odontoblast differentiation and later by differentiated odontoblasts (Vaahtokari *et al.*, 1991). Furthermore, TGF-β1 has been detected in dentin (Finkelman *et al.*, 1990). These authors suggested that the carious process which leads to the demineralization of dentin probably allows the release of biologically active molecules such as IGF, TGF-β or BMP. These molecules might stimulate odontoblasts during reactionary dentinogenesis.

Reparative dentinogenesis (distinct from reactionary dentinogenesis), implicates the differentiation of precursor cells present in the dental papilla. When studying the origin of such odontoblasts, Yamamura (1985) suggested two mechanisms which involved either pulpal cells close to the damage site, or odontoblast-precursor cells which migrated from deep in the dental pulp. However, the nature of the "stem cells" and the mechanism of their induction still remain open questions.

Attempts have been made to identify markers for the odontoblast lineage, however, immunological approaches and the production of monoclonal antibodies have not allowed identification of such a molecule yet (Aubin, 1985; Thesleff *et al.*, 1991; Zidan and Ruch, 1989). The question of the heterogeneity of neural crest-derived dental cells is still open and the use of both histo-cytological and functional criteria remains the best way to characterize the phenotype of functional odontoblasts.

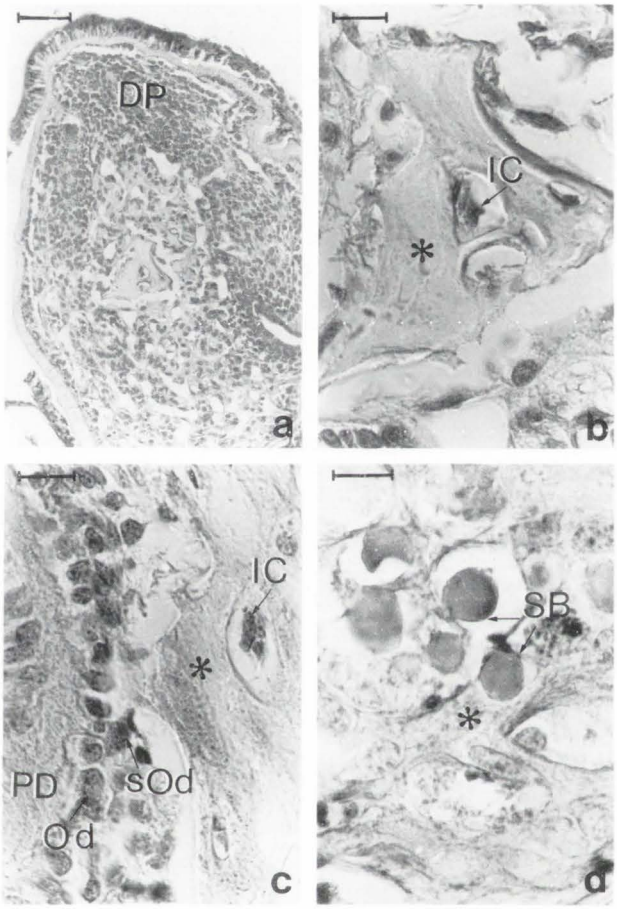

Figure 4. Histological sections of day-18 tooth germs cultured for 6 days (**c**) or 15 days (**a, b, d**) after microinjection of either dentin fraction able to initiate the terminal differentiation of odontoblasts (**a-c**) or TGF-β1 (**d**). The micro-needles (15-20 μm of diameter) were introduced from the opposite side of cusps to the middle of the dental papillae (DP) and the injection site was visualized either by the addition of Trypan Blue to the injected components solubilized in RPMI-1640 or by using Superose beads (SB). In all the cases, the response of the dental papillae cells consisted in an important accumulation of extracellular matrix (*) without any polarized deposition (a-d). Injection of active fraction of dentin close to the subodontoblastic cells (sOd) did not allow the polarization of the cells (c). In all cases (a-d), included cells (IC) were observed in the extracellular matrix. PD: predentin; Od: odontoblasts. Bars = 70 μm (a), 15 μm (b, c), and 10 μm (d).

There is no direct evidence to indicate that fundamentally different mechanisms control the cytological reorganization which characterizes odontoblast differentiation during odontogenesis and the reparative process. Although differences in cell shape can often be observed between odontoblasts involved in reparative dentinogenesis and those actively secreting primary dentin matrix, modulation in odontoblast morphology throughout their life-cycle is well recognized (Couve, 1986; Fox and Heeley, 1980; Romagnoli *et al.*, 1990; Takuma and Nagai, 1971). Odontoblast morphology appears to be closely related to functional activity. A question raised by Ten Cate (1985) concerned the epithelially-derived signal(s) which control(s) odontoblast differentiation during odontogenesis and the apparent lack of this signal in repair situation. Ruch (1985) suggested that during the reparative processes, fibrodentin could control odontoblast differentiation and thus play the role of the basement membrane during odontogenesis. More recently, the results obtained by *in situ* hybridization (Vaahtokari *et al.*, 1991) suggested that TGF-β1 which is synthesized by the inner dental epithelium at a stage where odontoblasts differentiate during odontogenesis, is later synthesized by the differentiated odontoblasts. Such a molecule might also be involved in a process triggering other dental papillae cells, which possess the necessary cell-surface receptors, to change their phenotype and to give rise to odontoblasts involved in reparative dentinogenesis. This possibility was reinforced by the biochemical analysis of dentin showing the presence of several growth factors (Finkelman *et al.*, 1990).

Reparative dentinogenesis has been studied in adult teeth and implicates dental papillae cells with a developmental history very different from that of the physiological preodontoblasts in developing teeth. Preodontoblasts from day-17 mouse molars and day-18 mouse molar dental papillae cells are probably much more closely related; for this reason, preliminary microinjection experiments were performed in order to investigate whether constituents, able to initiate the terminal differentiation of preodontoblasts, might also be able to initiate a similar process when interacting with dental papillae cells. Although day-18 dental papillae cells, which might comprise putative "progenitor odontoblasts", could be stimulated or activated to secrete large amounts of extracellular matrix, cell polarization was never observed. The microinjection of active components (dentin extracts or TGF-β) has just the same effect as TGF-β alone on dental papillae cultured in agar. Diffusion of the molecule throughout the mesenchymal tissue appears incompatible with achieving cell-polarization. The process of polarization might require the interaction of the active component with a restricted domain of the cell-surface, which would explain the positive effects of TGF-β immobilized with heparin (Bègue-Kirn *et al.*, 1992). For that reason, the approach of Tziafas *et al.* (1992a), who used Millipore filters coated with active molecule(s) implanted *in vivo*, might represent an alternative protocol.

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Discussion with Reviewers

J.E. Aubin: Currently, the major known mechanism by which microfilaments are affected by extracellular matrix is through integrin receptors. The section on polarization and fibronectin is somewhat confusing given the authors earlier discussion on the apparent integrin independence of the events.

Authors: A monoclonal antibody, MC16A16, directed against the 165 kDa protein impaired the initiation of odontoblasts differentiation and had no effect on the maintenance of polarized odontoblasts, which agrees with the transitory expression of the antigen at the apical pole of the differentiated cells. When added to the medium of cultured dental cells, this antibody was found to interfere with the organization of microfilaments although it had no effect on microtubules. Actually, we do not know how the interaction of the 165 kDa protein with the microfilament system is mediated? α-Actinin and talin, which have been reported to interact with the β1 subunit of integrins, and vinculin, have been purified and tested for their affinity for the 165 kDa protein transferred to PVDF. None of these proteins were found to interact directly in these conditions. One possibility is that the effect of the 165 kDa protein on microfilament organization is indirect.

J.E. Aubin: Since it is now known that RGD-independent integrin interaction can occur, it seems inappropriate to conclude that the odontoblast differentiation signal is integrin-independent. Please comment.

Authors: We have tried to be cautious about this point and our conclusion was not that odontoblast elongation and polarization were integrin-independent but RGD-independent processes. Our experiments with anti-integrin antibodies are too limited, when considering the number of known integrins, to exclude a possible participation of

these membrane constituents in the process of odontoblast differentiation.

J.E. Aubin: Please clarify whether the BMP-2 was also tried with heparin.
Authors: BMP-2 has not yet been tested in the presence of heparin but experiments should be done rapidly now.

M. Goldberg: The authors show that two basement membranes may appear in their experimental conditions. Can they further comment on this finding? Does this imply that either a first basement membrane (BM) was not destroyed because of a lack of collagenolytic enzymes and then a new BM was secreted? Does this mean that the inner dental epithelium cells moved from one place to another and built a new BM near the first one still present or is it a combination of the two hypotheses? This phenomenon is seen in some cases in the BM of incisor between capillaries and the outer enamel epithelium, but only in a few experimental conditions.
Authors: The duplication of the basement membrane was only observed as a consequence of the presence of GRGDS peptides which inhibited the interaction of preameloblasts with the basement membrane, leading to a detachment of this BM from the cell membrane. Then preameloblasts, which apparently require an interaction with a basement membrane component, deposited a new one which interacted with the basal cell surface of preameloblasts by means of RGD-independent mechanism. Indeed, the maintenance of duplicated segments of basement membrane suggests that proteolytic degradation processes, if existing, were very discrete at this stage.

M. Goldberg: For me, it is clear that tertiary dentin secreted by surviving true odontoblasts, post-mitotic cells, beneath a calcio-traumatic line, deeply differs from the material secreted by pulp cells, known to divide and migrate from the center of the pulp to the periphery (fibroblasts or pericytes or STEM cells together with endothelial cells?). This secretion is either seen as true orthodentin or osteodentin or any kind of mineralized tissue. It is the result of cells which cannot be called odontoblasts but neo-odontoblasts. What is the criterium to call these cells odontoblasts or odontoblast-like cells? Have the authors identified really a specific marker? For example, it seems according to some authors, that pulp cells as odontoblasts are able to synthesize and secrete phosphoproteins. Pulp cells cultured in spleen are able to form osteodentin (Ishizeki *et al.*, 1990). In other words, is there any specific marker of differentiation of these cells into new odontoblast? Is their polarization related to their new phenotype or only to the fact that placed along a solid surface, the secretion contribute to the thickening of the dentin or beads or any suitable material, in a more passive way than suggested by the paper?
Authors: This question raises some very fundamental points regarding the nature and activity of the cells associated with secretion of tertiary dentins. It also highlights the considerable confusion which exists within the literature concerning terminology. In response to an appropriate stimulus, such as dental caries, secretion of a tertiary dentin matrix is commonly seen. This dentin matrix may be laid down by either surviving post-mitotic true odontoblast cells or by a new generation of what are commonly referred to as odontoblast-like cells derived from the pulpal population of cells. The exact derivation of these latter cells is still a matter of debate (as discussed in the review), but they have been suggested to differentiate from the pool of undifferentiated mesenchymal cells, fibroblasts, pericytes and endothelial cells. The morphology of the cells responsible for secretion of this tertiary dentin matrix can be quite variable, as can be the structure of the dentin matrix itself. A commonly held view is that the tissue becomes more dysplastic with stronger stimuli and that the tubular nature of the dentin matrix becomes more irregular. The terms reactionary and reparative dentins have been widely used to describe this tertiary dentin, but unfortunately these terms have often been used synonymously. We would strongly propose that it would be appropriate to set out some definitions for these terms to avoid further confusion in this field as follows:

Reactionary dentin: A tertiary dentin matrix secreted by surviving post-mitotic odontoblast cells in response to an appropriate stimulus.

Reparative dentin : A tertiary dentin matrix secreted by a new generation of odontoblast-like cells in response to an appropriate stimulus, eventually after the death of the original post-mitotic odontoblasts responsible for primary and physiological secondary dentin secretion.

It is recognized that both reactionary and reparative dentin will each cover a wide spectrum of tissue morphologies dependent on the degree of tissue dysplasia. These points raise the question as to what specific markers can be used to identify odontoblasts and the matrix that they secrete. It has been suggested that phosphoproteins may represent markers of odontoblast phenotypic expression, but their considerable species variations, tissue distribution differences and variable reactivity amongst cells of the pulp-dentin complex must put this in question. At the present stage, it would appear that the best markers of odontoblasts are the morphologies of the cells and the tubular matrices that they secrete. It is apparent, however, that for the tertiary dentins and the cells involved in its secretion, such morphology must represent a broad spectrum as might be expected in any pathological situation. Nevertheless, this does not detract from the valuable information that can be gained from study of these processes to deepen our understanding of odontoblast differentiation and dental tissue repair.

A last point concerned polarization wondering, whether it could be a passive process. The experimental data reported in this review showed that, although it is probably an important parameter, interaction of odonto-

blasts with a solid surface was not sufficient to allow either the initiation or the maintenance of polarized odontoblasts. In some way, the elongation and polarization of these cells require specific conditions allowing also the functionality (i.e., activation of the metabolism) of the cells.

H. Magloire: This review clearly reveals the use in the literature of confusing terms such as secondary, tertiary, reparative, and reactionary dentins, then raising the lack of information concerning the origin of the cells involved, and consequently the process of differentiation. Some comments should be made: is the composition of secondary dentin (elaborated all along the life when the cells are differentiated) different to primary dentin?
Authors: It is difficult to answer such a question. Due to the low relative amount of secondary dentin, no biochemical data is available and the immunological investigations are restricted to small numbers of constituents.

H. Magloire: Is reactionary dentin very close to sclerotic dentin?
Authors: No biochemical analysis is available but these two types of dentin probably result from different processes. Dentin sclerosis represents increased deposition of peritubular dentin and possibly hypermineralization of intertubular dentin. Reactionary dentin is laid down at the pulp-dentin interface and its appearance suggests a more limited maturation of the deposited extracellular matrix.

H. Magloire: Pulp response to carious process or cavity preparation has always been associated with a very localized inflammatory process, not necessary situated in front of the injured dentin because of the tubules course. So the appreciation of impaired (or not) odontoblasts is clear after observation of serial slices of the whole tooth.

Also, because of the high degree of differentiation as well as the evident diameter of tubules (i.e., cell processes), it seems unlikely that odontoblasts injured by drilling could be alive for a long time. So I propose to the authors not to be so affirmative.
Authors: Observations were performed on serial sections of teeth and indicated that odontoblast response (i.e., reactionary dentin deposition) occurred at the ends of tubules in contact with the cavity and that odontoblast death did not occur at these sites in unexposed cavities.

These topics were not introduced in detail in the present paper since they are being discussed in a paper submitted by Smith *et al*, (Odontoblast stimulation by dentine matrix components, Archs. oral Biol.).

A kinetic study has been performed and the data allow us to maintain what is written here: odontoblasts do survive and are indeed stimulated.

M. Goldberg: Do the authors have any comments on the Höhl cells and their role(s) during these differentiating processes?

Authors: The peripheral, dentin-forming sector of the pulp is generally divided into three zones: the outer odontoblast zone, the medial cell-poor zone of Weil, and the inner cell-rich zone of Höhl (Höhl, 1896). These Höhl cells appear in terms of composition of their organelles to be almost identical with odontoblasts but differ from the latter by their predominantly bipolar arrangements. Clearly, they seem to be a population of cells distinct from the general pulpal fibroblasts and may be regarded as a pool of undifferentiated mesenchymal progenitor cells. It seems probable that they may be derived from the group of mesenchymal daughter cells, which after the final cell division prior to odontoblast terminal differentiation, can be seen as a layer of sub-odontoblast cells because of the perpendicular orientation of the mitotic cells to the basement membrane giving rise to superimposed daughter cells. As such, these cells will have experienced entirely the same developmental events as the odontoblasts with the exception of the final inductive signal for terminal differentiation. It would thus seem probable that these cells would be eminently suitable candidates for giving rise to the new generation of odontoblast-like cells during reparative dentinogenesis after receipt of a suitable inductive trigger, both in terms of their position in the pulp as well as their developmental history.

Editor: Please provide definitions of GRGDS, GRGDS(P), RGD and GRGES.
Authors: These symbols correspond to the sequence of amino acids according to the international code and means the following:
GRGDS: glycine-arginine-glycine-aspartic-serine; GRGDS (P): glycine-arginine-glycine-aspartic-serine (pro); RGD: arginine-glycine-aspartic; and GRGES: glycine-arginine-glycine-glutamic-serine.